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Impact of exposure duration by low molecular weight compounds on interferon-γ and interleukin-4 mRNA expression and production in the draining lymph nodes of mice

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Abstract

The local lymph node assay (LLNA) is used to identify allergens by means of dermal exposure. For hazard identification, besides identification also the distinction between contact and respiratory allergens is of importance. We have previously shown that a modified LLNA can be used to identify respiratory allergens, on the basis of Con A induced IL-4 production. Here we show a good qualitative correlation between mRNA expression and production of IFN-γ and IL-4. This suggests that distinction between contact and respiratory allergens may also be studied at the mRNA expression level. Secondly, another assay, similar to the modified LLNA but differing in the duration and the number of allergen applications as well as in the ex vivo culture conditions, here denoted as 'longer' assay, has been reported to be able to identify contact allergens, on the basis of (spontaneous) IFN-γ production. In the present study we have compared these assays. Similar to our previous findings, in the modified LLNA exposure to the respiratory allergen trimellitic anhydride (TMA) resulted in a ~ 10-fold higher Con A induced IL-4 production compared with the contact allergen dinitrochlorobenzene (DNCB), while exposure to both allergens resulted in a similar Con A induced IFN-γ production. In the 'longer' assay, TMA exposure resulted in Con A induced IL-4 production whereas DNCB exposure did not. Importantly, only a 2-fold higher spontaneous IFN-γ production was induced by DNCB compared with TMA, the difference being not statistically significant. Thus, although the 'longer' assay indeed showed a somewhat higher IFN-γ induction by DNCB compared with TMA, the magnitude and robustness of this effect question its applicability. These results favor the modified LLNA since it is shorter, and combines identification of allergens (by cell proliferation) with identification of respiratory allergens (by IL-4 production). Compounds that induce cell proliferation with a low concomitant IL-4 production may thus be identified as contact allergens, although the need to positively identity such allergens remain.

Keywords: Local lymph node assay; IFN-γ; IL-4; Dinitrochlorobenzene; Trimellitic anhydride; mRNA; Protein

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1. Introduction

An assay to identify contact allergens in mice by means of dermal exposure is the local lymph node (LN) assay (LLNA; Basketter et al., 2002; Gerberick et al., 2000; Ryan et al., 2000). This assay measures proliferation of the draining LN cells, and comprises a sensitizing phase only. Induction of a 3-fold or higher proliferative response compared with vehicle treated controls identifies a test chemical as sensitizer. The LLNA has been endorsed by Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) as a stand-alone method for skin sensitization hazard identification. Experimentally this assay is also used to identify respiratory allergens. For hazard identification, besides identification of allergens also the distinction between contact and respiratory allergens is of importance. It has been proposed that contact allergens preferentially induce a T-helper 1 (TH1) response, whereas respiratory allergens preferentially induce a T-helper 2 (TH2) response (Dearman et al., 1992). These responses can be distinguished on the basis of ex vivo cytokine production by the draining LN cells, such as IFN-γ, that is produced by TH1 cells, and IL-4, that is produced by TH2 cells. We have developed a modified LLNA, that differs from the LLNA in (i) the ^{3}H-labeling procedure (ex vivo vs. in vivo in the LLNA); (ii) the strain of mice used (BALB/c vs. female CBA in the LLNA); and (iii) the time point of LN harvesting (days 1–5 vs. day 1 in the LLNA). The rationales for these alterations are (i) the reduction in radioactive waste. We have a firm belief that this alteration does not affect the performance of the assay, and (ii) the general use of BALB/c mice in experimental studies. When cytokine measurement is included, the modified LLNA has the advantages that cell proliferation and cytokine production can be measured in the same cells, which is likely to provide more reliable data, and that it requires less experimental animals. We have previously shown within this modified LLNA that mRNA expression of IFN-γ and IL-4 in (intact) draining LN, and also IFN-γ production by Con A stimulated LN cells could not be used to identify contact allergens (as being distinct from respiratory allergens; Vandebriel et al., 2000). Failure to use IFN-γ mRNA expression for identifying contact allergens was confirmed by Hayashi et al. (2001). In contrast, IL-4 production by Con A stimulated LN cells was able to identify respiratory allergens (as being distinct from contact allergens).

Gene expression profiling is a powerful tool to screen for differences between T-cell subsets (Chtanova et al., 2001; Hamalainen et al., 2001; Rogge et al., 2000). As a first step to implement such techniques in our studies we wished to establish differences in cytokine mRNA expression that underlie already established differences in cytokine production. Therefore, the first aim of the present study was to analyze IFN-γ and IL-4 mRNA expression in Con A stimulated LN cells, so in cells similarly activated as the ones analyzed previously for cytokine production.

Another, experimentally used assay in mice, similar to the modified LLNA but differing in the duration and number of applications (days -12, -7, -2, -1, and 0, compared with days -2, -1, and 0 in the modified LLNA) is also based on dermal exposure (Dearman et al., 1994, 1995, 1996a,b). It comprises both a sensitizing and effector phase. In this assay it has been shown that contact allergens can be identified on the basis of ex vivo IFN-γ production, while respiratory allergens can be identified on the basis of ex vivo IL-4 production. The culture conditions required to identify contact allergens (48–96 h at 10^7 cells/ml in the absence of Con A) are different from the culture conditions to identify respiratory allergens (24 h at 10^6 cells/ml in the presence of Con A). Since we were unable to positively identify contact allergens in the modified LLNA, the second aim of the present study was to test the positive identification of contact allergens using different regimes of applications and different ex vivo cell culture conditions.

2. Methods

2.1. Chemicals

The chemicals used were 2,4-dinitrochlorobenzene (DNCB; 98% purity; Sigma-Aldrich, Zwijndrecht, the Netherlands) and trimellitic anhydride

(TMA; 97% purity; Sigma-Aldrich). The chemicals were dissolved in 4:1 acetone/olive oil (AOO). The final concentration was 1% weight /volume (w/v) for DNCB and 10% (w/v) for TMA. AOO was used as vehicle control.

2.2. Animals

BALB/c mice (obtained from our own breeding colony, or from Harlan/CPB, Zeist, the Netherlands) were used at the age of 6–8 weeks. In each separate experiment, either male or female mice were used. The diet consisted of ground standard laboratory chow (RMH-B, Hope Farms, Woerden, the Netherlands). Food and water were given ad libitum. The breeding colony of the animals was pre-screened/monitored for endogenous pathogenic viruses and bacteria and was found negative.

2.3. Sensitization

'Short' assay (modified LLNA): For 3 consecutive days, 25 μl of 1% DNCB, 10% TMA, and AOO was applied to the dorsum of both ears. At 5 days after the last application, the local (auricular) LN were excised and weighed. Results are expressed as mean weight (mg) ± S.E.M. 'Longer' assay: At days 0 and 5, 50 μl of 1% DNCB, 10% TMA, and AOO was applied to each of the shaved flanks. At days 10, 11, and 12, 25 μl of 1% DNCB, 10% TMA, and AOO was applied to the dorsum of both ears. At 5 days after the last application, the local (auricular) LN were excised and weighed. Results are expressed as mean weight (mg) ± S.E.M.

Combined assay: At days 0 and 5, 50 μl of 1% DNCB, 10% TMA, and AOO was applied to each of the shaved flanks. At days 10, 11 and 12, 25 μl of 1% DNCB, 10% TMA and AOO was applied to the dorsum of both ears. Alternatively, only at days 10, 11 and 12, 25 μl of 1% DNCB, 10% TMA and AOO was applied to the dorsum of both ears. At 1 and 3 days after the last application, the local (auricular) LN were excised and weighed. Results are expressed as mean weight (mg) ± S.E.M.

2.4. Cell suspensions

The culture medium used was RPMI-1640 (Gibco, Grand Island, NY) supplemented with 10% heat inactivated Fetal Calf Serum (PAA, Linz, Austria), 100 μg/ml streptomycin, and 100 IU/ml penicillin. Cell suspensions were made by pressing the LN through a cell strainer (Falcon, Franklin Lakes, NJ). Cells were counted using a Coulter Counter (Coulter Electronics, Luton, UK).

2.5. Lymphocyte proliferation test

Two$*10^6$ cells in 200 μl culture medium were seeded in triplicate wells of U-bottom 96-well microtiter plates (Greiner, Frickenhausen, Germany), and 37 kBq methyl-^{3}H-thymidine (specific activity 18.5 GBq/mmol; Amersham, Little Chalfont, UK) in 10 μl PBS was added. LN cells from allergen treated animals were seeded for individual animals; four animals were used, resulting in four cell cultures. In case of AOO treated animals, however, LN cells were seeded from two pooled animals, because the number of LN cells obtained per animal was insufficient to perform the subsequent assays; six animals were used, resulting in three cell cultures. The plates were incubated at 37 °C in a humidified atmosphere containing 5% CO_2. After 24 h the cells were harvested on glass-fiber filters (LKB-Wallac, Espoo, Finland) using a multiple cell culture harvester (LKB-Wallac). ^{3}H-incorporation was measured using liquid scintillation counting. Results are expressed as the median of triplicates, in mean cpm per well (cpm per 2×10^6 cells) ± S.E.M. LN cell proliferation on a per animal basis is the parameter of choice to identify allergens. Hence, results are also calculated and expressed per animal, as mean (cpm) ± S.E.M.

In caseof AOO treated animals, the mean cell number of the two pooled animals was multiplied by the ^{3}H-incorporation of the cell culture obtained from these pooled animals, to express the cell proliferation data on a per animal basis.

2.6. Cell culture

The same suspensions as described above were cultured at 10^6 cells/ml culture medium with Con A (5 μg/ml) and 5×10^{-5} M 2-mercaptoethanol in flat-bottom 12-well culture plates (Costar, Cambridge, MA) at 37 °C in a humidified atmosphere containing 5% CO_2 for 24 h. After culture the cells were spun down and the supernatants were pipetted off. The cell pellets were frozen on dry ice and stored at −70 °C until use (PCR). Aliquots of the supernatants were frozen on dry ice and stored at −70 °C until use (ELISA).

In some experiments, the same suspensions as described above were cultured at 10^7 cells/ml culture medium in the absence of Con A for 72 h. Other culture conditions were kept unchanged.

2.7. RNA isolation

RNA was isolated using the SNAP total RNA isolation kit (Invitrogen, San Diego, CA) according to the manufacturer's instructions. All steps were performed at room temperature (RT), except if indicated. Centrifugation steps were performed at 13 000 rpm. Briefly, 2 volumes (vol; 1 vol = 300 μl) of lysis buffer (5.3 M guanidine–HCl, 1.5% Triton-X100, 2.5 mM Tris–HCl, pH 7.5, 0.25 mM EDTA) was added to the LN cell pellets. The LN cell pellets were then homogenized by pipetting, and 1 vol isopropanol was added. After mixing the sample was transferred to a column. The column was centrifuged for 1 min and 1 vol wash solution (100 mM NaCl) was added. The column was centrifuged again for 1 min and again 1 vol wash solution was added. The column was centrifuged for 2 min. After adding 0.225 vol water to the column and 5 min incubation, RNA was eluted by centrifugation for 1 min. After DNase treatment for 10 min at 37 °C, 0.75 vol binding buffer (7 M guanidine–HCl, 2% Triton-X100) was added and mixed. Then 0.5 vol isopropanol was added. After mixing the sample was transferred to a fresh column. The column was washed as described above. After adding 0.07 vol water to the column and 5 min incubation, RNA was eluted by centrifugation for 1 min. The RNA concentration was measured spectrophotometrically at 260 nm. RNA was subjected to electrophoresis on a 1% agarose gel and visualized by ethidium bromide staining. RNA was used only when both 28 and 18 S rRNA bands were intact. Samples were stored at −70 °C until use.

2.8. cDNA synthesis

One microgram RNA was reverse transcribed using the Reverse Transcription System (Promega, Leiden, the Netherlands). Briefly, 10.5 μl water containing 1 μg RNA was mixed with 0.5 μl oligo (dT) primer (500 μg/ml) and incubated for 10 min at 70 °C. Samples were then incubated at 4 °C for at least 5 min. To the mixture, 4 μl $MgCl_2$ (25 mM), 2 μl 10*RT-buffer, 2 μl dNTP (10 mM each), 0.5 μl RNasin (50 U/μl) and 0.5 μl AMV-RT (30 U/μl) was added and incubated at 42 °C for 1 h. The reaction was stopped by heating the samples for 5 min at 72 °C. Samples were stored at −20 °C until use.

2.9. Polymerase chain reaction

The PCR mixture contained 5 μg/ml of 5′-primer, 5 μg/ml of 3′-primer, 1.5 mM $MgCl_2$, 200 μM of each of the nucleotides dATP, dCTP, dGTP and dTTP (Roche, Almere, the Netherlands), and 2 U AmpliTaq Gold DNA polymerase (Perkin–Elmer, Nieuwerkerk a/d IJssel, the Netherlands) in 1*PCR buffer II (Perkin–Elmer). The primers used were: IFN-γ, 5′ GCT CTG AGA CAA TGA ACG CT and 3′ AAA GAG ATA ATC TGG CTC TGC (229 bp), IL-4, TCG GCA TTT TGA ACG AGG TC and 3′ GAA AAG CCC GAA AGA GTC TC (216 bp), and HPRT 5′ GTC AAG CAG TAC AGC CCC AAA ATG G and 3′ TAG TGC AAA TCA AAA GGG ACG CAG C (385 bp). Primers were synthesized by GibcoBRL (Life Technologies, Breda, the Netherlands). HPRT was used as a housekeeping control. One and 2 μl cDNA was amplified as follows: incubation for 12 min at 94 °C, then 24, 27, and 30 cycles (HPRT), or 27, 30, and 33 cycles (IFN-γ and IL-4), each of 45 s at 94 °C, 45 s at 55 °C, and 2 min at 72 °C, and a final incubation for 7 min at 72 °C. PCR products were electrophorized on a 2% agarose gel and visualized by ethidium bro-

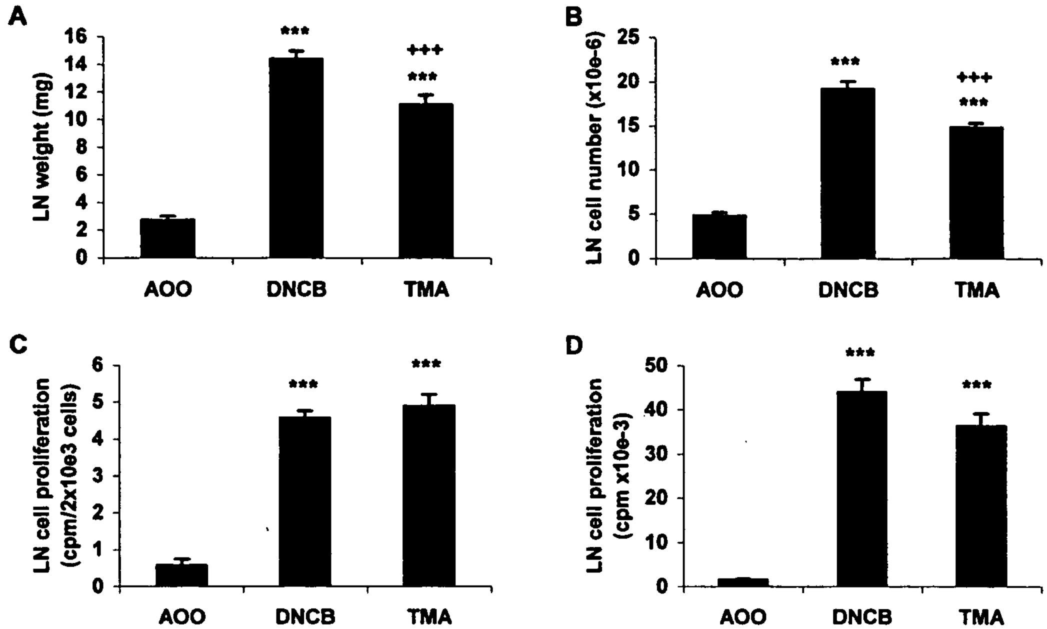

Fig. 1. Effect of topical application on LN weight (A), LN cell number (B), and LN cell proliferation per culture (C) and per animal (D). Mice were treated by topical application on both ears with DNCB, TMA, or AOO as vehicle control for 3 consecutive days. Five days after the last application, the local LN were excised, pooled (per animal for allergen treatment and per two animals for vehicle treatment), and cell suspensions were prepared. Cells were cultured for 24 h in the presence of ^{3}H-thymidine. ^{3}H-incorporation was measured. Number of animals = 4, except for AOO where this number = 6. Thus, for AOO the number of data points (n) = 6 in Figs. A and B, and $n = 3$ in Figs. C and D, whereas for allergen treatment $n = 4$ in Figs. A–D. Results are expressed as LN weight (mg) ± S.E.M. (A), as LN cell number (× 10^6) ± S.E.M. (B), as cpm/2 × 10^6 cells ± S.E.M. (C), and per animal values were calculated by multiplying the ^{3}H-incorporation by the LN cell number, and expressed as cpm ± S.E.M. (D); (***), significantly different from AOO ($P < 0.001$). (+ + +), significantly different from DNCB ($P < 0.001$).

mide staining. The intensities of the PCR products were quantified using a Gel Doc 1000 (BioRad, Veenendaal, the Netherlands) and Molecular Analyst software (BioRad). The intensities of the PCR products were plotted against the intensities of a molecular mass ladder (BioRad), and regression analysis was performed. Using the regression curve the amount of PCR product was calculated. Results are expressed as mean amount of PCR product (ng) ± S.E.M.

2.10. ELISA

IFN-γ and IL-4 concentrations were determined by ELISA. Briefly, 96-well plates (Nunc-Immuno Plate, Roskilde, Denmark) were coated with 1 μg/ml anti-mouse IFN-γ (R4-6A2, rat IgG$_1$; Pharmingen, San Diego, CA) in coating buffer (0.04 M carbonate buffer, pH 9.6). After overnight incubation at 4 °C the plates were incubated in blocking buffer (1% bovine serum albumin (BSA; Sigma, Axel, the Netherlands) plus 0.05% Tween-20 (Merck, Amsterdam, the Netherlands), in PBS) for 2 h at 37 °C and washed (0.05% Tween-20). Recombinant mouse IFN-γ (Biosource, Camarillo, CA) was used as a standard. Standard as well as serial dilutions of culture supernatants were added to the plate. Plates were incubated at 37 °C for 2 h and washed. Biotinylated anti-mouse IFN-γ (0.5 μg/ml; XMG 1.2, rat IgG$_1$; Pharmingen) was added and incubated for 1 h at RT. The plates were washed, and poly horseradish peroxidase labeled streptavidin (10 000-fold dilution, Strepta-E+, Central Laboratory of the Blood transfusion service, Amsterdam, the Netherlands) was added and incubated for 1 h at RT. Plates were washed again and TMB solution (0.1 mg/ml TMB (Sigma) plus 0.006% H_2O_2, in 0.1 M NaAc, pH 5.5) was

added. The plates were read at 450 nm. For IL-4, a similar protocol was used, with 0.5 μg/ml anti-mouse IL-4 (11B11, rat IgG_1; Pharmingen) for coating, recombinant mouse IL-4 (Peprotech, Rocky Hill, NJ) as a standard, and 0.5 μg/ml biotinylated anti-mouse IL-4 (BVD6-24G2, rat IgG_1; Pharmingen) for detection. Antibodies, standards, samples, and streptavidin were diluted in 0.5% BSA plus 0.05% Tween-20, in PBS (Van Halteren et al., 1997). Detection limits are 16 μg/ml for IFN-γ and 8 pg/ml for IL-4. The results are expressed as mean (pg/ml) ± S.E.M. Similar to cell proliferation, results are also expressed per two LN (per animal), i.e. the concentration (pg/ml) is multiplied by the LN cell number, divided by the cell culture concentration (10^6 per ml), and expressed as mean (pg) ± S.E.M.

2.11. Statistics

Statistical analysis was performed using the two-tailed Student *t*-test.

3. Results

3.1. 'Short' assay (modified LLNA)

3.1.1. Lymphocyte proliferation

Application of DNCB and TMA resulted in a significantly increased LN weight compared with AOO. DNCB application resulted in a significantly higher LN weight compared with TMA (Fig. 1A). Similar observations were made for the LN cell number (Fig. 1B). Application of DNCB and TMA resulted in a significantly increased ^{3}H-incorporation compared with AOO, both per culture (Fig. 1C) and per two LN (per animal; Fig. 1D). DNCB and TMA induced a similar ^{3}H-incorporation.

3.1.2. Cytokine mRNA expression

Application of DNCB and TMA resulted in a significantly ($P < 0.001$) higher amount of PCR product of the housekeeping gene HPRT compared with AOO. Application of DNCB and TMA resulted in similar amounts of HPRT PCR product (results not shown).

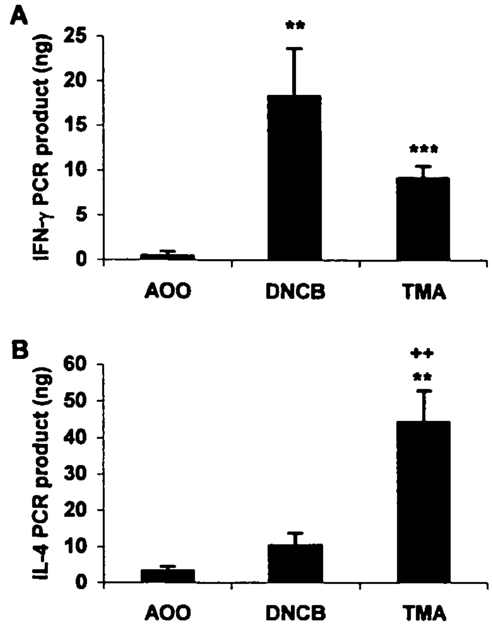

Fig. 2. Effect of topical application on IFN-γ mRNA expression (A) and IL-4 mRNA expression (B). Mice were treated by topical application on both ears with DNCB, TMA, or AOO as vehicle control for 3 consecutive days. Five days after the last application, the local LN were excised, pooled (per animal for allergen treatment and per two animals for vehicle treatment), and cell suspensions were prepared. Cells were cultured for 24 h in the presence of Con A. The cells were pelleted, and analyzed for cytokine mRNA levels by RT-PCR. Number of animals = 4, except for AOO where this number = 6. Thus, for AOO the number of data points (n) = 3, whereas for allergen treatment $n = 4$. Results are expressed as the amount of PCR product (ng) of IFN-γ (A) and IL-4 (B), ±S.E.M. (**) and (***), significantly different from AOO ($P < 0.01$ and < 0.001, respectively). (+ +), significantly different from DNCB ($P < 0.01$).

Application of DNCB and TMA resulted in a significantly higher amount of IFN-γ PCR product compared with AOO. DNCB application resulted in a 2-fold higher amount of IFN-γ PCR product compared with TMA (Fig. 2A). This difference was, however, not statistically significant.

DNCB application resulted in a more than 3-fold increase in the amount of IL-4 PCR product compared with AOO (Fig. 2B). This effect was, however, not statistically significant. TMA application resulted in an almost 14-fold increase in the amount of IL-4 PCR product compared with AOO. Importantly, TMA application resulted in

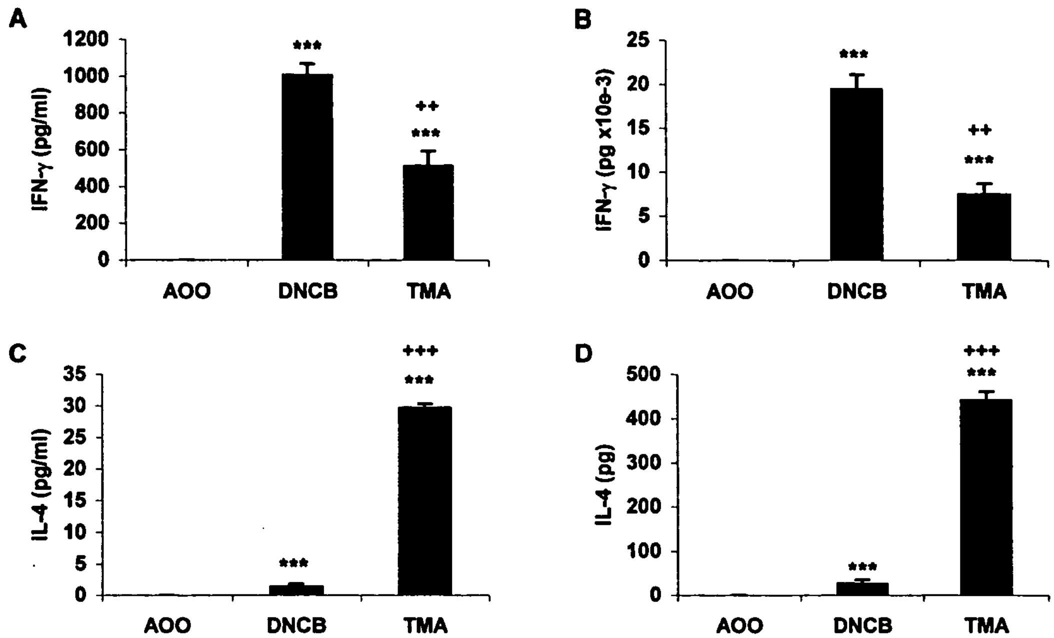

Fig. 3. Effect of topical application on IFN-γ production per culture (A) and per animal (B), and on IL-4 production per culture (C) and per animal (D). Mice were treated by topical application on both ears with DNCB, TMA, or AOO as vehicle control for 3 consecutive days. Five days after the last application, the local LN were excised, pooled (per animal for allergen treatment and per two animals for vehicle treatment), and cell suspensions were prepared. Cells were cultured for 24 h in the presence of Con A. Cells were pelleted and the resulting supernatants were analyzed for cytokine content using ELISA. Number of animals = 4, except for AOO where this number = 6. Thus, for AOO the number of data points (n) = 3, whereas for allergen treatment $n = 4$. Results are expressed as pg/ml ± S.E.M. (A and C), and per animal values were calculated by multiplying the cytokine concentration by the LN cell number, and expressed as amount of cytokine (pg) ± S.E.M. (B and D); (***), significantly different from AOO ($P < 0.001$). (+ +) and (+ + +), significantly different from DNCB ($P < 0.01$ and $P < 0.001$, respectively).

a (statistically significant) 4-fold increase in the amount of IL-4 PCR product compared with DNCB.

3.1.3. *Cytokine production*

Application of DNCB and TMA induced IFN-γ production, whereas (Fig. 3A and B). DNCB application resulted in a (statistically significant) 2–3-fold higher IFN-γ production compared with TMA.

Application of DNCB and TMA induced IL-4 production, (Fig. 3C and D). Importantly, TMA application resulted in a (statistically significant) 17–23-fold higher IL-4 production compared with DNCB.

3.2. *'Longer' assay*

3.2.1. *Lymphocyte proliferation*

Application of DNCB and TMA resulted in a significantly increased LN weight and LN cell number compared with AOO (Fig. 4A and B). Application of DNCB and TMA resulted in a significantly increased ^{3}H-incorporation compared with AOO, both per culture (Fig. 4C) and per two LN (per animal; Fig. 4D). TMA application induced a somewhat higher increase in LN weight, LN cell number, and ^{3}H-incorporation compared with DNCB, but these differences were not statistically significant.

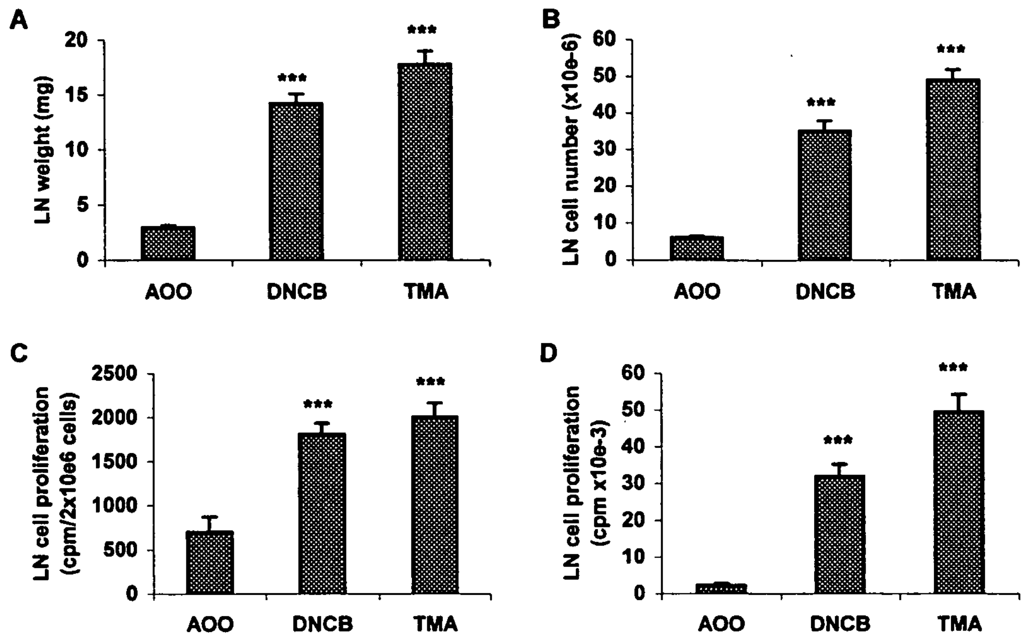

Fig. 4. Effect of topical application on LN weight (A), LN cell number (B), and LN cell proliferation per culture (C) and per animal (D). Mice were treated by topical application on both ears with DNCB, TMA, or AOO as vehicle control at days 0, 5, 10, 11 and 12. Five days after the last application, the local LN were excised, pooled (per animal for allergen treatment and per two animals for vehicle treatment), and cell suspensions were prepared. Cells were cultured for 24 h in the presence of ^{3}H-thymidine. ^{3}H-incorporation was measured. Number of animals = 4, except for AOO where this number = 6. Thus, for AOO the number of data points (n) = 6 in Figs. A and B, and $n = 3$ in Figs. C and D, whereas for allergen treatment $n = 4$ in Figs. A–D. Results are expressed as LN weight (mg)$\pm$S.E.M. (A), as LN cell number ($\times 10^{-6}$)$\pm$S.E.M. (B), as cpm/2 $\times 10^6$ cells$\pm$S.E.M. (C), and per animal values were calculated by multiplying the ^{3}H-incorporation by the LN cell number, and expressed as cpm$\pm$S.E.M. (D); (***), significantly different from AOO ($P < 0.001$).

3.2.2. Cytokine mRNA expression

Application of DNCB and TMA resulted in a significantly ($P < 0.001$) higher amount of HPRT PCR product compared with AOO. Application of DNCB and TMA resulted in similar amounts of HPRT PCR product (results not shown).

Application of DNCB and TMA resulted in a significantly higher amount of IFN-γ PCR product compared with AOO. Application of DNCB and TMA resulted in similar amounts of IFN-γ PCR product (Fig. 5A).

Application of DNCB and TMA resulted in a significantly higher amount of IL-4 PCR product compared with AOO (Fig. 5B). Importantly, TMA application resulted in a (statistically significant) more than 6-fold higher amount of IL-4 PCR product compared with DNCB.

3.2.3. Cytokine production

Application of DNCB and TMA clearly induced IFN-γ production, whereas the LN of the AOO treated animals did not show production of this cytokine (Fig. 6A and B). DNCB and TMA similarly induced IFN-γ production.

Application of DNCB and TMA induced IL-4 production, whereas the LN of the AOO treated animals showed only marginal production of this cytokine (Fig. 6C and D). Importantly, TMA application resulted in a (statistically significant) 7–11-fold higher IL-4 production than DNCB.

3.3. "Combined" assay

To directly compare the 'short' assay and the 'longer' assay they were combined in single experiments. These experiments compared (i)

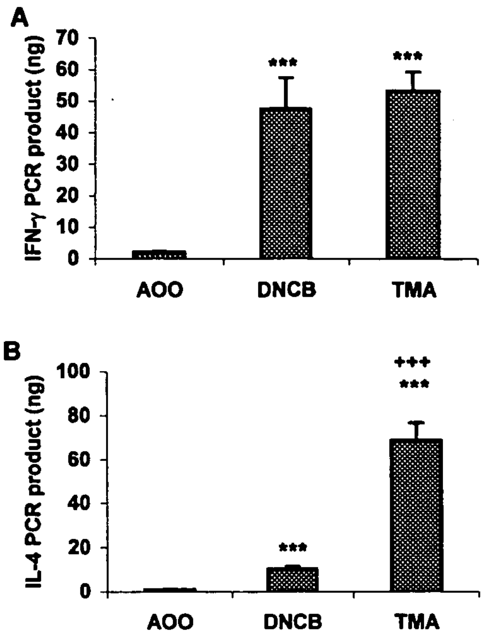

Fig. 5. Effect of topical application on IFN-γ mRNA expression (A) and IL-4 mRNA expression (B). Mice were treated by topical application on both ears with DNCB, TMA, or AOO as vehicle control at days 0, 5, 10, 11 and 12. Five days after the last application, the local LN were excised, pooled (per animal for allergen treatment and per two animals for vehicle treatment), and cell suspensions were prepared. Cells were cultured for 24 h in the presence of Con A. The cells were pelleted, and analyzed for cytokine mRNA levels by RT-PCR. Number of animals = 4, except for AOO where this number = 6. Thus, for AOO the number of data points (n) = 3, whereas for allergen treatment $n = 4$. Results are expressed as the amount of PCR product (ng) of IFN-γ (A) and IL-4 (B), ±S.E.M. (***), significantly different from AOO ($P < 0.001$). (+ + +), significantly different from DNCB ($P < 0.001$).

'short' and 'longer' exposure; (ii) sacrificing the mice 1 and 3 days after final exposure; and (iii) two ex vivo culture conditions (10^6 cells/ml for 24 h in the presence of Con A, to 10^7 cells/ml for 72 h in the absence of Con A). Using either exposure protocol and day of sacrificing the mice (a) application of DNCB and TMA resulted in a significantly increased LN weight and LN cell number compared with AOO ($P < 0.001$; not shown), (b) application of DNCB and TMA resulted in a significantly increased ^{3}H-incorporation compared with AOO, both per culture and per animal; ($P < 0.001$; not shown), and (c) DNCB and TMA induced a similar LN weight and LN cell number, and a similar ^{3}H-incorporation (not shown).

Tables 1 and 2 show a representative experiment analysing the induction of IFN-γ and IL-4 production by DNCB and TMA. From the 8 protocols used, the highest ratio of IFN-γ production induced by DNCB compared to TMA was 1.97, with none of the protocols resulting in a statistically significant difference. The highest ratio was obtained after "longer" exposure, sacrificing the mioce at 1 day after final application and *ex vivo* culture of 10^7 cells/ml for 72 h in the absence of Con A (protocol F). In a replicate experiment, the highest ratio of IFN-γ production induced by DNCB compared to TMA was 1.88 (again using protocol F), with again none of the protocols resulting in a statistically significant difference (results not shown). Thus, none of the protocols tested was capable of identifying DNCB on the basis of IFN-γ production. All protocols that employ Con A stimulated cells showed a higher induction of IL-4 production by TMA compared to DNCB ($P < 0.001$). Thus, the "short" assay were similarly capable of identifying TMA on the basis of IL-4 production.

4. Discussion

The present study confirms our previous findings (Vandebriel et al., 2000) that in a modified LLNA, TMA induces a much higher IL-4 production compared with DNCB. The 2- to 3-fold higher IFN-γ production induced by DNCB compared with TMA in this assay suggests that IFN-γ production may be used for positive identification of contact allergens. In our view, however, the relatively small magnitude of this ratio, and the lack of reproducibility precludes IFN-γ production from being used to positively identify contact allergens within the modified LLNA.

We have previously shown that in intact LN IFN-γ mRNA expression was not induced by DNCB or TMA application (compared with AOO; Vandebriel et al., 2000). Furthermore, IL-4 mRNA expression was similarly induced by these two allergens. In the present study IFN-γ

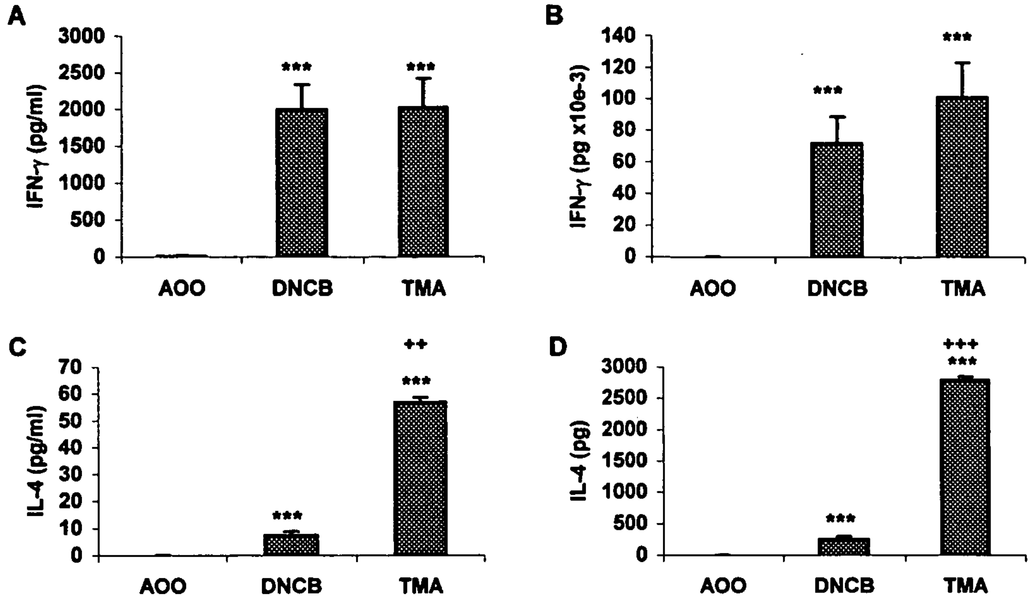

Fig. 6. Effect of topical application on IFN-γ production per culture (A) and per two LN (per animal; B), and on IL-4 production per culture (C) and per animal (D). Mice were treated by topical application on both ears with DNCB, TMA, or AOO as vehicle control at days 0, 5, 10, 11, and 12. Five days after the last application, the local LN were excised, pooled (per animal for allergen treatment and per two animals for vehicle treatment), and cell suspensions were prepared. Cells were cultured for 24 h in the presence of Con A. Cells were pelleted and the resulting supernatants were analyzed for cytokine content using ELISA. Number of animals = 4, except for AOO where this number = 6. Thus, for AOO the number of data points (n) = 3, whereas for allergen treatment $n = 4$. Results are expressed as pg/ml ± S.E.M. (A and C), and per animal values were calculated by multiplying the cytokine concentration by the LN cell number, and expressed as amount of cytokine (pg) ± S.E.M. (B and D). (***), significantly different from AOO ($P < 0.001$). (+ +) and (+ + +), significantly different from DNCB ($P < 0.01$ and $P < 0.001$, respectively).

Table 1
Induction of IFN-γ production by DNCB and TMA in the 'short' assay (modified LLNA) and the 'longer' assay

# Applications[a]	Sacrifice[b]	Culture[c]	DNCB[d]	TMA[d]
A -2/-1/0	1	$24+10^6$	4.6±0.4	5.1±0.5
B -2/-1/0	1	$72-10^7$	4.0±0.6	4.2±0.2
C -2/-1/0	3	$24+10^6$	4.0±0.2	4.0±0.4
D -2/-1/0	3	$72-10^7$	3.3±0.4	3.6±0.1
E -12/-7/-2/-1/0	1	$24+10^6$	2.0±0.3	1.8±0.1
F -12/-7/-2/-1/0	1	$72-10^7$	1.6±0.8	0.9±0.2
G -12/-7/-2/-1/0	3	$24+10^6$	1.7±0.3	1.3±0.1
H -12/-7/-2/-1/0	3	$72-10^7$	1.4±0.4	1.2±0.2

Expressed in pg × 10^{-3} per ml; the detection limit was 0.016×10^3 pg/ml.

[a] Compounds were topically applied on the flanks at days -12 and -7 (when indicated), and on the ears at days -2, -1, and 0.

[b] Mice were sacrificed 1 or 3 days after the final application.

[c] Cells (10^6 per ml) were cultured for 24 h in the presence (+) of Con A. Alternatively, cells (10^7 per ml) were cultured for 72 h in the absence (−) of Con A.

[d] 1% DNCB or 10% TMA was applied. $N = 4$.

and IL-4 mRNA expression are measured in Con A stimulated LN cells. The ratio of IFN-γ mRNA expression induced by DNCB versus TMA of 2.0 is in reasonable agreement with the corresponding ratio of IFN-γ production, being two per culture and three per animal. Remarkably, while the ratio of IL-4 mRNA expression induced by TMA versus DNCB is 4.3, the corresponding ratio of IL-4 production is 17 per culture and 23 per animal. Thus, while these ratios are relatively similar for IFN-γ, for IL-4 production the ratio is four to five times higher compared with IL-4 mRNA expression. The reason for this difference is unknown. In summary, in contrast to cytokine mRNA expression in intact LN, in Con A stimulated LN cells cytokine mRNA expression profiles are qualitatively similar to profiles of cytokine production. The clear difference in IL-4 mRNA expression induced by TMA versus DNCB suggests that parameters for positive identification of contact

Table 2
Induction of IL-4 production by DNCB and TMA in the 'short' assay (modified LLNA) and the 'longer' assay

# Applications[a]	Sacrifice[b]	Culture[c]	DNCB[d]	TMA[d]
A -2/-1/0	1	$24+10^6$	91 ± 13	271 ± 10 (***)
B -2/-1/0	1	$72-10^7$	ND	31 ± 7
C -2/-1/0	3	$24+10^6$	66 ± 3	207 ± 8 (***)
D -2/-1/0	3	$72-10^7$	ND	44 ± 7
E -12/-7/-2/-1/0	1	$24+10^6$	79 ± 13	332 ± 6 (***)
F -12/-7/-2/-1/0	1	$72-10^7$	ND	18 ± 16
G -12/-7/-2/-1/0	3	$24+10^6$	44 ± 7	242 ± 8 (***)
H -12/-7/-2/-1/0	3	$72-10^7$	ND	21 ± 4

Expressed in pg/ml; ND, not detectable; the detection limit was 8 pg/ml.
[a] Compounds were topically applied on the flanks at days -12 and -7 (when indicated), and on the ears at days -2, -1, and 0.
[b] Mice were sacrificed 1 or 3 days after the final application.
[c] Cells (10^6 per ml) were cultured for 24 h in the presence (+) of Con A. Alternatively, cells (10^7 per ml) were cultured for 72 h in the absence (−) of Con A.
[d] 1% DNCB or 10% TMA was applied; (***), significantly different from DNCB ($P < 0.001$). $N = 4$.

allergens (other than IFN-γ) may be discovered by methods that rely on mRNA expression measurement. Using C57BL/6 but not BALB/c mice, Hayashi et al. (2001) showed that IL-12p40 mRNA expression was indeed able to identify contact allergens. Although of relevance, the strain dependence of this parameter may limit its applicability. Recently, DNA arrays were used to identify differentially induced genes in assays relatively similar to the LLNA. Of 6500 genes analyzed, and after confirmation by RT-PCR, (only) expression of 'monokine induced by IFN-γ', a member of the platelet factor four family of cytokines, was induced significantly more by the contact allergen oxazolone than by the respiratory allergen toluene diisocyanate (He et al., 2001). Furthermore, of 8734 genes analyzed expression of 'onzin' and 'guanylate-binding protein-2' were upregulated 48 h after application of the contact allergen dinitrofluorobezene (Betts et al., 2002). Northern blotting and/or RT-PCR confirmed these findings over a more extensive time course.

An assay that not only positively identifies respiratory allergens, but also contact allergens has been developed by Dearman et al. (1994, 1995) Dearman et al. (1996a,b). This assay, here denoted as 'longer' assay, is similar to the modified LLNA, except for the number and duration of applications: three applications (on days 0, 1, and 2) in the modified LLNA, and five applications (on days 0, 5, 10, 11, and 12) in the 'longer' assay. In addition, the ex vivo culture conditions differ in that for measuring IFN-γ (but not IL-4) production (i) a 10-fold higher cell concentration is used, (ii) Con A is omitted, and (iii) the duration of culture is considerably longer (48–96 h). Since the positive identification of contact allergens, not achieved so far using the modified LLNA, poses an advantage of the 'longer' assay, we chose to evaluate this latter assay. In our hands, under conditions of similar stimulation by DNCB and TMA, TMA was able to induce a more than 6-fold higher IL-4 mRNA expression and a 7- to 11-fold higher IL-4 production compared with DNCB. IFN-γ mRNA expression and production were, however, similar for DNCB and TMA. This suggests that in our hands the 'longer' assay has no added value compared with the 'short' assay with respect to the identification of contact versus respiratory allergens. Plitnick et al. (2002) recently showed that in the 'longer' assay DNCB did not induce IFN-γ mRNA expression, whereas TMA induced mRNA expression of IL-4, and also IL-10 and IL-13. This again suggests that the 'longer' assay is able to achieve similar results compared with the 'short' assay, but has no added value compared with the 'short' assay.

To make a more definitive comparison between both assays, combined experiments were undertaken. In this comparison also alternative culture

conditions were tested. These conditions have been reported to identify contact allergens on the basis of IFN-γ production when used in conjunction with 'longer' exposure (Dearman et al., 1994, 1995, 1996a,b). Both assays were able to identify TMA on the basis of IL-4 production. Exposure protocol F, used by Dearman et al. (1994, 1995, 1996a,b) to identify contact allergens on the basis of IFN-γ production, resulted in the highest ratio of induction of IFN-γ production by DNCB compared with TMA. However, this ratio was only 2-fold and not statistically significant. Thus, the magnitude and robustness of this effect question its applicability. Manetz et al. (2001) found that in a modified LLNA (with 24 h incubation ex vivo with Con A), TDI induced an 8.3-fold higher IL-4 mRNA expression and a 2.5-fold lower IFN-γ mRNA expression than DNFB. Using the 'longer' assay (with similar ex vivo culture conditions) TDI induced a 35-fold higher IL-4 mRNA expression and a 2.5-fold higher IFN-γ mRNA expression than DNFB, suggesting that at least under these culture conditions the 'longer' assay is not useful to positively identify DNFB.

In conclusion, based on the results presented here the modified LLNA ('short' assay) is preferred since it is shorter, and combines both identification of sensitizera (by cell proliferation) and identification of respiratory sensitizers (by IL-4 production). Induction of cell proliferation and concomitantly low IL-4 induction may identify contact allergens. Moreover, when performing dose response studies the modified LLNA combines both potency assessment and discrimination between contact and respiratory allergens (Van Och et al., 2002). Although contact allergens may be identified that way, it cannot be excluded that this process will benefit from the availability of a positive marker. Methods based on analyzing mRNA expression, such as DNA arrays, have the potential to find such markers.

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